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Quantification of natural and synthetic glucocorticoids in calf urine following different growth-promoting prednisolone treatments

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ABSTRACT

Over the last few years, low levels of prednisolone have been reported in several cattle urine samples by a number of laboratories within the EU at an average concentration of 2.0 ng mL^{-1}. The occurrence of prednisolone residues together with increased levels of hydrocortisone and cortisone in urine and tissue samples of untreated animals seems to demonstrate that traces of this steroid can be produced endogenously during stressful situations. Therefore, the endogenous origin of prednisolone makes difficult to correlate positive samples to a potential illicit treatment. An experimental study was developed to investigate the presence of natural and synthetic glucocorticoids and to evaluate levels of excreted prednisolone following growth-promoting treatments. Urine samples from calves undergone oral treatment with prednisolone, alone and in association with dexamethasone, were analyzed by a LC–MS/MS method, validated according to the Commission Decision 2002/657/EC. We also investigated if urinary free 6β-hydroxyhydrocortisone/hydrocortisone ratio could be a reliable biomarker of illicit treatment with prednisolone and dexamethasone in calves. Our data revealed that urinary levels of prednisolone after both oral prednisolone treatments, never exceeded the value of 1.1 ng mL^{-1}. Similar prednisolone levels were found in urine samples of untreated calves. Moreover the presence of 6β-hydroxyhydrocortisone below the CCα value made possible to estimate the 6β-hydroxyhydrocortisone/hydrocortisone ratio only in a very limited number of samples. Obtained data suggest that further criteria have to be considered to allow correct decisions about the urinary presence of prednisolone during control activities.

1. Introduction

Prednisolone is a glucocorticoid widely used as a therapeutic agent in veterinary practice for the treatment of a wide range of metabolic diseases, allergic and inflammatory diseases in food-producing animals [1–5]. Due to the potential toxic effects that prednisolone and other glucocorticoid residues can exert on meat consumers, their use in livestock is strictly regulated with withdrawal periods between treatment and slaughtering and maximum residue limits (MRLs) established in edible biological matrices and milk. The MRL values for prednisolone therapeutic use are set as follows: 4.0 μg kg^{-1} for muscle and fat, 6.0 μg kg^{-1} for milk and 10.0 μg kg^{-1} for liver and kidney [6]. Despite the ban on the use of glucocorticoids for purposes or under conditions other than those laid down in the Community legislation (Council Directive 2003/74/EC) [7], the administration of these substances for growth-promoting purposes is still practiced within the European Union [8], because of their well-known capacity to increase weight gain and to reduce the feed conversion rate [9,10]. The number of positive samples for prednisolone is increased over the last few years and low levels of prednisolone have been reported in bovine urines by a number of EU laboratories at average concentration of 2.0 ng mL^{-1}. The presence of this glucocorticoid in urine could originate from weak collection/storage of samples or microbial contamination from faecal origin [11,12]. Furthermore, the occurrence of prednisolone residues, together with increased levels of hydrocortisone and cortisone, in both urine and tissue (muscle, liver, kidney) samples of untreated animals [13], seems to demonstrate that traces of this steroid can be also endogenously produced, especially under stressful situations [11–18]. Therefore, the naturally occurrence of prednisolone together with continuous evolution of illicit treatments makes difficult to correlate positive samples to an eventual illicit treatment.

Dexamethasone is another glucocorticoid available in veterinary medicine, and it still remains the most extensively used compound for the treatment of metabolic and inflammatory disorders in ruminants [19]. Although its use is intended for therapeutic

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purpose, dexamethasone is being increasingly used in cattle fattening, alone or in association with other drugs. The MRLs established for therapeutic use of dexamethasone are: 2.0 μg kg^{-1} for liver, 0.75 μg kg^{-1} for muscle, 0.75 μg kg^{-1} for kidney and 0.3 μg kg^{-1} for milk [7]. Dexamethasone is known to be a cytochrome P4503A (*CYP3A*) inducer in human and rat. CYP3A is an important enzyme family metabolizing both drugs and endogenous substances. It also metabolizes hydrocortisone to 6β-hydroxyhydrocortisone which has been used as an indicator of CYP3A induction in man [20–22]. Since hydrocortisone shows a pronounced diurnal variation, the ratio of free 6β-hydroxyhydrocortisone to free hydrocortisone in urine is used rather than 6β-hydroxyhydrocortisone itself in order to correct for daily and inter-individual variations in adrenal cortisol production [20]. It is likely that the hepatic metabolism of hydrocortisone to 6β-hydroxyhydrocortisone catalyzed by the CYP3A also occurs in cattle. Nevertheless only few data on the urinary 6β-hydroxyhydrocortisone/hydrocortisone ratio in cattle are available but the results presented by Capolongo et al. [23] seem to suggest it as new promising screening test for detection of long-term dexamethasone illicit oral administration in cattle.

An experimental study was developed to investigate the presence of natural and synthetic glucocorticoids in calf urine and to evaluate levels of excreted prednisolone following two different prednisolone growth-promoting treatments. We also investigated if urinary free 6β-hydroxyhydrocortisone/hydrocortisone ratio could be a reliable biomarker of illicit treatment for calves treated with prednisolone in association with dexamethasone. Analyses were performed by using a multi-residue LC–MS/MS method validated according to the Commission Decision 2002/657/EC [24] with simultaneous quantification of dexamethasone, prednisolone, prednisone, hydrocortisone, cortisone and 6β-hydroxyhydrocortisone.

2. Experimental

2.1. Animals and experimental protocol

The experimental plan was designed according to the guidelines of the European Union law for care and use of experimental animals (Council Directive 86/609/EEC recognized and adopted by the Italian Government (D.L. 27/01/1992 No. 116)) [25] and the study was approved by the Ministry of Health and the Ethics Committee for animal welfare of the University of Turin.

Nineteen Friesian male veal calves were raised for 6 months under controlled experimental conditions in multiple pens. They were fed a diet available on the market, usually employed in zootechnical practice with *ad libitum* access to water. Feed ingredients were dairy-products, oils and fats, oilseed products and by-products, cereal products and by-products and minerals (21.5% proteins, 20% fats, 0.3% fiber and 7.5% ash). During the seventh month of the experimental trial, they were divided into three groups and underwent two different prednisolone growth-promoting treatments: 5 animals underwent dexamethasone plus prednisolone treatment (0.2 mg of dexamethasone 21-disodium phosphate + 4 mg of prednisolone, both orally administered per capita/day for 20 consecutive days), other 10 animals underwent prednisolone treatment (8 mg of prednisolone orally administered per capita/day for 20 consecutive days), the remaining 4 animals were used as controls for both groups of treated calves.

Appropriate measures were taken to avoid any kind of cross-contamination between the three different groups of animals.

2.2. Samples collection

Urine samples were collected (taking care to prevent faecal contamination) after milk administration and waiting for spontaneous micturition and were immediately stored in the dark at −20 °C until analysis.

Samples from dexamethasone plus prednisolone treated calves: urine samples were collected before the first administration, then at the 3rd, 7th, 14th, 20th, 21st, 22nd, 23rd and 28th. The last samples were collected directly from the bladder after slaughtering (30th day).

Samples from prednisolone treated calves: urine samples were collected before the first administration then at 3rd, 7th, 14th, 20th, 21st, 22nd, 23rd, 28th day. The last samples were collected directly from the bladder after slaughtering (31st day).

Samples from control calves: urine samples were collected in the same sampling days of both treated calf groups.

2.3. Chemicals and reagents

All solvents were HPLC or analytical grade and purchased from Riedel-de Haën (Seelze, Germany). Water was purified by Milli-Q System (Millipore, Bedford, MA, USA). Sodium acetate anhydrous and β-glucuronidase–arylsulphatase (*Helix pomatia*) were obtained from Merck (Darmstadt, Germany), this latter was used as supplied. Sodium hydroxide was purchased from J.T. Baker (Deventer, Netherlands) and acetic acid were obtained from Sigma–Aldrich (St. Louis, MO). OASIS HLB SPE cartridges (3 mL, 60 mg) were supplied by Waters (Milford, MA, USA). Dexamethasone, prednisolone, and hydrocortisone were provided by Riedel-de Haën whereas prednisone, cortisone and 6β-hydroxyhydrocortisone were purchased from Sigma–Aldrich (St. Louis, MO). Triamcinolone acetonide-d_6 (internal standard, IS) was provided by RIKILT, the European Union Reference Laboratory in Wageningen, Netherlands.

The 0.1 M acetate buffer solution (ABS) was prepared by dissolving 12.3 g of sodium acetate anhydrous in 800 mL of purified water: the pH was adjusted to 4.8 and then water was added to the final volume of 1000 mL.

The 1 M NaOH solution was prepared by dissolving 40.0 g of sodium hydroxide in 1000 mL of purified water. The 1% (v/v) acetic acid solution was prepared by dissolving 10.0 mL of acetic acid in 1000 mL of purified water.

2.4. Standard solutions

A 1 mg mL^{-1} stock solution of each standard was prepared in methanol; from these solutions, a 10 μg mL^{-1} dilution was prepared in methanol. A mixture of all standards is then prepared at dilutions of 10 ng mL^{-1} and 100 ng mL^{-1}. These working standard solutions were stored at 4 °C and prepared on a daily basis.

2.5. Samples preparation

2.5.1. Extraction of dexamethasone, prednisolone, prednisone, hydrocortisone and cortisone

The analytical procedure for extraction and purification of urine samples has been described in our previous works [13,14]. This procedure required β-glucuronidase–arilsulfatase enzyme solution (*H. Pomatia*) for enzymatic digestion and purification by OASIS HLB SPE cartridge. The use of the *H. Pomatia* enzyme allows to estimate the overall (free and conjugate forms) glucocorticoid concentration in urine.

2.5.2. Extraction of free 6β-hydroxyhydrocortisone, hydrocortisone and cortisone

The procedure reported in the Section 2.5.1 is not suitable for 6β-hydroxyhydrocortisone extraction from urine probably because of the relative higher polarity of this compound compared to hydrocortisone and cortisone. Thus, the procedure described by Hu et al. [22] with minor modification was used as described